AD_____

Award Number: DAMD17-99-1-9148

TITLE: Breast Cancer Therapeutics, Environmental Estrogens, and the Estrogen Receptor (ER); Characterization of the Diverse Ligand Binding Properties of the ER

PRINCIPAL INVESTIGATOR: Deborah S. Wuttke, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado
Boulder, Colorado 80309-0572

REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Final (1 Jul 1999 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Breast Cancer Therapeutics, Environmental Estrogens, and the Estrogen Receptor (ER); Characterization of the Diverse Ligand Binding Properties of the ER

5. FUNDING NUMBERS
DAMD17-99-1-9148

6. AUTHOR(S)
Deborah S. Wuttke, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado
Boulder, Colorado 80309-0572

E-Mail: Deborah.Wuttke@Colorado.EDU

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct form its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer. In addition, a variety of compounds introduced into the environment by human activity have also been found to act as estrogen mimics and alter reproductive function and development. The goals of this project are to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. Towards this end, we have expressed and purified the ligand binding domain of the estrogen receptor, and studied complexes with estradiol, the natural ligand, and tamoxifen, a partial antiestrogen in use as a breast cancer therapeutic using high-resolution heteronuclear NMR spectroscopy.

14. SUBJECT TERMS
Breast cancer, estrogen receptor, nuclear magnetic resonance spectroscopy, estradiol tamoxifen

15. NUMBER OF PAGES
30

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct from its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer (*i.e.,* tamoxifen). In addition, a variety of compounds introduced into the environment as a result of human activity have also been found to act as estrogen mimics and alter reproductive function and development. The estrogenic behavior of these compounds has proven difficult to predict from their structures; at first glance many of these hormone mimics bear little structural resemblance to natural estrogens. The molecular level details of the conformational changes that allow the ER to tightly bind a diverse array of compounds and result in diverse patterns of gene activation are not understood and yet are critical to assessing and predicting the behavior of potentially estrogenic compounds. The goals of this project were to develop high resolution strategies to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. A complete structural understanding of how various ligands interact with the ER, and are able to elicit different responses, will assist in identifying compounds with therapeutic benefit for treating breast cancer.

BODY

The large numbers of compounds that are able to bind and activate the ER necessitates the use of an innovative and rapid approach. We began our investigation of ligand binding to the ER using multidimensional nuclear magnetic resonance spectroscopy (NMR) studies of complexes of these compounds with the ligand binding domain (LBD) of the ER. The ER-LBD is uniformly isotopically labeled with ^{13}C and/or ^{15}N and ligand binding is followed by acquiring heteronuclear single quantum coherance (HSQC) spectra. These fingerprint like spectra are exquisitely sensitive to molecular conformation. Once this strategy is established, we proposed to study the structural effects of several point mutations isolated from breast cancer cell lines. Analysis of our NMR data was significantly aided by the present availability of the crystal structures of the ER bound to both estrogens and antiestrogens. The use of NMR spectroscopy as an efficient tool for screening for compounds with estrogenic or antiestrogenic activity, or with activity specific to either ERα or ERβ was explored.

Progress towards the tasks in the statement of work are as follows:

Technical Objective 1:

Prepare and purify unlabeled LBD-ERα
Identify solution conditions suitable for NMR experiments
Collect and evaluate preliminary homonuclear NMR data

The tasks associated with technical objective 1 as proposed were completely achieved. The LBD-ER was prepared by expression in BL21(DE3) *E. coli* by induction with IPTG from a pET plasmid. Complete purification was achieved using an estradiol affinity column that afforded protein of high purity and activity in one step (Figure 1). In addition, the purified protein is highly stable to proteolysis, a problem that rendered protein purified by other strategies inactive within a few days of preparation. A complete buffer screen revealed optimal NMR buffer conditions (20mM d-Tris, 200mM NaCl, 1mM d-DTT, 0.02% sodium azide) that allowed us to prepare samples with concentrations of up to 500 μM. Homonuclear NMR data obtained on these samples revealed features consistent with a protein of the anticipated molecular weight. The optimum temperature for data collection was determined to be 30°C. The anticipated molecular weight of the estradiol bound complex was confirmed by light scattering data.

The tasks associated with technical objective 1 were mostly achieved within the first year of the grant. However, for a variety of reasons we needed to expand this objective to develop more reliable and flexible protein expression and purification strategies. Therefore, in the second year of the grant, we extensively re-evaluated the conditions for protein expression. We discovered that our previous protocol did not reliably produce protein, *i.e.*, in certain growths when protein expression was induced at high ODs no protein production was observed. This is presumably due to loss of ampicillin resistance at high bacterial cell concentration. This is not uncommon, as the resistance to antibiotic is conferred by a secreted protein. Thus, induction at an OD of 1.0 at 600 nm was discovered to give completely reliable protein production. We modified all of our protocols to accommodate this improvement.

We also realized that, in order to study the environmental estrogens that bind the ER-LBD much more weakly than the natural ligand estradiol, we would need to produce a recombinant ligand-free form of the ligand binding domain (LBD) of the estrogen receptor. Although our estradiol affinity column provides a very pure ER-LBD, elution from the column requires competition with a ligand of similar affinity (*e.g.* tamoxifen or estradiol itself). For weak binding ligands, this protocol would be very inefficient as most ligands are not soluble at the concentrations that are needed to compete the protein from the column. Thus, a stable, ligand-free preparation protocol was needed. Development of this protocol has the added benefit of allowing characterization *in vitro* of the ligand free form by NMR.

Technical Objective 2:

Prepare and purify ligand complexes of ^{15}N-labeled ER from minimal media growths
Collect and evaluate ^{15}N-^{1}H HSQC NMR data at 500 MHz
Collect and evaluate ^{15}N-^{1}H HSQC NMR data at 600 MHz
Prepare and purify ligand complexes of ^{13}C,^{15}N-labeled ER from minimal growths
Collect and evaluate ^{13}C and ^{15}N-^{1}H HSQC NMR data at 500 MHz
Collect and evaluate ^{13}C and ^{15}N-^{1}H HSQC NMR data at 600 MHz
Prepare and purify ligand complexes of isotopically labeled ER from minimal growths
Collect and evaluate ^{13}C and ^{15}N-^{1}H HSQC NMR data of complexes at optimum NMR conditions

Investigate deuteration strategies

The tasks associated with technical objective 2 have been completely achieved. Growth of uniformly ^{15}N labeled protein progressed smoothly based on the expression and purification scheme developed for unlabeled protein in Technical Objective 1. ^{15}N-^{1}H HSQC NMR data were collected at 500 and 600 MHz (Figure 2). Significant improvement of data quality was observed at higher field strengths and with the addition of TROSY pulse sequences. However, even with the addition of TROSY to improve resolution, the large size of the complex (56 kDa) and it's helical structure (which typically results in less dispersion) contributes to a quality of NMR data that will make it quite difficult to make complete resonance assignments as planned (Figure 3). Based on these data, we determined that data should be collected at the highest possible magnetic field strength, as success on complexes this size has been reported at 800 and 900 MHz. This led us to aggressively pursue funding opportunities for the creation of a high field NMR facility at the University of Colorado (see below). A tamoxifen-bound complex was prepared to compare tamoxifen to estradiol-bound forms of the protein Figure 4). Significant chemical shift differences were noted between these two complexes, as anticipated, illustrating the validity of our strategy. Interestingly, the tamoxifen bound form consistently gave higher quality spectra. Thus this state of the protein is most suitable for high-resolution studies.

In order to interpret the chemical shifts in the context of available structures, resonance assignments need to be completed. Towards this end, uniformly ^{13}C, ^{15}N and ^{2}H labeled protein has been prepared. The ^{13}C and ^{15}N labels will allow assignment of the backbone resonances via their direct scalar couplings. Complete deuteration is employed to further reduce linewidths by eliminating the dominant mechanism of line broadening, relaxation via ^{1}H-^{1}H dipolar couplings. To achieve this result, growth in D_2O has been optimized. While yields are slightly reduced we clearly can obtain sufficient quantities for structural work. Our improved protein expression protocol has significantly enhanced our ability to prepare these labeled samples, as the D_2O growths are very expensive.

Our optimized protocol for prepation of fulled deuterated, ^{13}C and ^{15}N labeled material follows: A single colony of transformed BL21(DE3)pLysS bacteria from a M9 minimal media plate was used to innoculate 1 mL M9 minimal media plus supplements containing $(^{15}NH_4)_2SO_4$, 3x glucose, 50 mg/mL carbenicillin (2x), 68 mg/mL chloramphenicol (2x), and made up in 70% D_2O. The culture was grown to saturation at 37 °C (over 17 hours). This culture was used to innoculate 10 mL of the same media, which was grown to an OD_{600} above 1.5, and then used to innoculate 100mL of media made up in 85% D_2O, with all the other same components. When the 85% D_2O culture reached an OD_{600} above 1.5 (approximately 10 hours), the shaker was cooled to 20 °C, and the 100mL culture was used to innoculated 1L of 99% D_2O media as above, except the $(^{15}NH_4)_2SO_4$ was increased to 1.5x. Increasing the glucose and $(^{15}NH_4)_2SO_4$ in the media increased yields. Adjusting the pH manually to 7.0 every 4-6 hours with 0.5 M NaOH made up in D_2O was tried during the growth. When the culture reached an OD_{600} of 1.0 (approximately 10 hours), ampicillin was added and the culture was induced with IPTG, as above, for 16-18 hours. Induction times ranging from 5 to 45 hours were tried, and 16-18 hours was found to produce optimal yields. The cells were then harvested as above. These growths

produced 13.5 mg purified ER-LBD per liter (Figure 5). The NMR data obtained on this sample are the best data we were able to acquire (Figure 6).

Extensive efforts were aimed during the second year of funding at obtaining triple resonance data for resonance assignments. Pilot triple resonance experiments, including the HNCA and HNCO, were collected, and every possible parameter was scrupulously optimized. However, the signal obtained on these experiments was not amenable to full resonance assignment. In fact, only about half of the anticipated resonance signals were observed. This is due to the large size of the ER-LBD, which is a symmetric dimer of molecular weight 56 kDa. Even with our advances using TROSY and deuteration, we have not been able to reduce the T2 relaxation of the complex by a significant enough amount to prevent complete decay during the time of the pulse sequence. Unfortunately, the other triple-resonance experiments that are needed to make assignments, the HNCACO, HNCACB, CBCACONH etc, are even longer so the relaxation issues associated with large (*i.e*, slowly tumbling systems) are even worse. Attempts to assign a partial data set such as this would be rather ill conceived, as it would be easy to make a mistake early in the assignment process that would lead to complete misassignment of the resonances. In fact, very few systems larger than 30 kDa have been assigned to date, highlighting the challenges associated with working with systems of this size. In our laboratory, we recently solved the structure of a 22 kDa protein with a 3.5 kDa DNA ligand. Even at this size, the system was a challenge at 600 MHz. All of the systems greater than 30 kDa have been assigned using field strengths larger than 750 MHz. These studies indicate that further optimization, either through preparation of higher concentration samples or data collection at higher field, are necessary. In addition, we began to pursue other strategies for investigating ligand recognition by the ER-LBD in the event that the NMR studies could not be completed.

Based on our preliminary NMR data collected for assignment, we recognized that data needed to be collected at as high of a field strength as possible. Significant improvement in sensitivity, signal-to-noise, and the TROSY enhancement are all anticipated when moving from 600 to 800 or 900 MHz. Towards this end we tried to establish collaborations with researchers with access to an 800 MHz spectrometer. While it may be possible to collect short data sets on spectrometers dedicated to other PIs, it has not been possible to obtain the quantities of instrument time needed for these studies. Routine access to high field spectroscopy on a regular basis is needed to complete this phase of the project. While the timeframe for establishing facilities of this type are beyond the time frame of this grant award, we are determined to continue this project, and decided to invest the effort needed to establishing the necessary facilities.

Creation of a 800 and 900 MHz NMR Facility

To gain routine access to high field NMR, we (University of Colorado Department of Chemistry and Biochemistry) established a consortium with the University of Utah Medical School and the University of Colorado Health Sciences Center to create a shared facility with an 800 MHz spectometer. The three participating institutions will share the time, operating costs and matching costs of the facility equally. Our proposal for this instrumentation was funded by the NIH/NSF Joint Shared Instrumentation program and, independently, at the Keck Foundation (DSW is coPI of the NIH/NSF grant). The instrumentation will be housed in Boulder in a

facility currently under renovation. We anticipate delivery of the 800 MHz spectrometer in Spring of 2004. Access to high field instrumentation will significantly enhance our ability to obtain full backbone resonance assignments on this challenging system.

We are delighted to report that in addition we have just been awarded funding for a 900 MHz NMR facility (DSW is PI of this grant). The University of Colorado was one of two institutions selected nationwide for this honor by the NIH. The facility will be housed in a specially designed and engineered facility custom built to accommodate the unique siting requirements of this instrument. The 900 MHz instrument will radically change our ability to work with systems the size of the ER (and beyond), and represents a net $8 million investment in NMR structural biology at our institution. The data obtained on the ER was a key component of our proposal justifying the need for enhanced capability.

New Studies Incorporated into this Project:

Based on our concern that it would be impossible to obtain full backbone resonance assignments as planned within the timeframe of the grant with our available resources, we expanded our efforts towards the understanding of ligand specificity of the ligand binding domain into new areas. These research projects have dramatically enhanced our understanding of the ER and will allow us to continue our studies in this important area. These research areas are described below.

Detergent Solubilization Protocol

We developed a differential detergent solubilization method to enhance our yield of refolded protein. The affinity column protocol described above, while effective in producing pure, stable protein, leads to losses of up to 50% of the ER-LBD that could potentially be recovered using an optimized recovery strategy. This becomes a concern when working with expensive, isotopically labeled samples. Thus, we developed the following detergent-based protocol. After expression of the LBD using the standard technique, the pellet is first washed with a buffer containing the detergent octyl glucoside which preferentially solubilizes almost all the other cell proteins. After octyl glucoside extraction, the cell pellet is washed with a buffer containing the detergent Zwittergent 3-12, which then solubilizes the estrogen receptor LBD.

We have established that this unusual procedure does indeed yield significant amounts of the ER-LBD. We have determined that, following dialysis of detergent, the LBD refolds and regains the ability to bind estrogen. The circular dichroism spectrum of material prepared by this method reveals the presence of helical structure. However, we could not obtain a concentrated sample of this for NMR analysis. In addition, preliminary attempts at titration of environmental ligands into a sample of the ER-LBD resulted in irreversible precipitation of the protein. Given the unusual nature of this protocol, we could not rule out residual effects of the detergent or incorrect protein folding as the cause of poor sample solubility. Therefore, we invested a significant effort in developing a more straightforward purification protocol for ligand-free ER-LBD.

Preparation and Characterization of the Ligand-free State of the Protein

In order to study weak binding ligands, we needed to develop a ligand-free form of the ligand binding domain (LBD) of the estrogen receptor. This will allow us to examine structural

differences between the free and bound forms and ultimately look at the amount of conformational change that occurs upon binding of the ligand. Our current purification strategy relied on an estradiol affinity column, and the protein necessarily is eluted with ligand. For weak binding ligands, this protocol will not work, as most ligands are not soluble at the concentrations that are needed to compete the protein from the column. Thus, a stable, ligand-free preparation protocol was needed.

We selected a His-tagged strategy for several reasons. First, the addition of a His tag on the protein provides a good handle for purification using a variety of readily commercially available columns and beads. Second, we engineered the His tag such that it could be readily cleaved off with thrombin, thus allowing removal of the tag if it interferes with either receptor activity or the quality of the NMR spectrum. Finally, this strategy broadens the types of approaches that we could use to add in the variety of ligands targeted in this study, as we can investigate ligand addition to both immobilized and free protein.

In order to prepare the His-tagged construct of the ER-LBD, we cloned the ER-LBD sequence (aa 297-554) into a pET 15b plasmid (Novagen) using the restriction enzyme BamH1. The integrity of the subcloning was assured by sequencing of the recombinant plasmid at the University of Colorado DNA sequencing facility. Conditions for protein expression were assessed using test inductions (see Figure 7) and purification was performed using standard protocols for His-tag purifications. After sonicating the cells twice and spinning down cell debris after each sonication, the supernatant was loaded on a Pharmacia chelating column that had been preloaded with nickel ions. The column was washed with a low level of imidazole (30 mM) to remove non-specifically bound proteins, and then the His-tagged ER-LBD was eluted with 500 mM imidazole. The resulting protein is >95% pure by Coomassie-stained SDS-PAGE gel, and stable to long term proteolysis. CD characterization of the ligand free ER-LBD revealed the presence of significant helical structure (Figure X). The protein is active in ligand binding as it fully binds our estradiol-affinity column. While there are still significant concerns regarding solubility, the His-tagged version of the ER-LBD should allow us to pursue structural studies with weakly binding environmental estrogens. Development of protocols for introduction of these ligands is currently in progress.

The ligand-free ER-LBD was studied by NMR. We obtained a sample of uniformly ^{15}N-labeled protein using the protocol described above. The protein could only be concentrated to 250 μM before precipitation, and the protein stuck non-specifically to all surfaces used in its preparation, both properties associated with unfolded/aggregated material. These features are manifest in the ^{15}N HSQC spectrum. While resonances can clearly be identified, the protein appears to adopt a partially unfolded character. The very strong resonances are likely due to the unstructured His-tag, and provide a reference for the completely unfolded state. The combination of the CD and NMR data on the protein indicate the presence of "molten globule" like state for the ligand-free form of the protein (Figure 8 and 9). The CD clearly reveals the the presence of regular secondary structure, in particular a high content of α-helical structure, while the NMR spectrum resembles a partially folded or nascently structured state. These data are consistent with the observation that the ligand-free state of the protein is bound to heat shock proteins *in vivo*. This phenomenon may be needed to confer either affinity or specificity for ligand binding, representing an important problem in molecular recognition.

Thermodynamic Studies

In addition to the NMR studies described above, CD denaturation studies have been conducted on the estradiol and tamoxifen complexes. These data have allowed us to compare the thermodynamic stabilities of these complexes, and we have determined that the midpoint for denaturation is very similar for these complexes. The midpoint for denaturation is ~5.5 M guanadinium hydrochloride (GdnHCl) for the estradiol-bound complex, and ~6 M GdnHCl for the tamoxifen complex (Figure 10 and 11). As expected from the known structures of the ER-LBD, the CD signal of the folded protein is entirely helical, transitioning to a typical unfolded CD spectrum as chaotropic concentration is increased. The unfolding transition is not a simple 2-state unfolding, and likely involves partially folded intermediates. This behavior is not unexpected for a dimeric protein of this size.

CD Characterization of Different Ligand Bound States of the Protein: Complexes with Weakly Bound Ligands

To investigate structural changes that occur in the ER-LBD upon binding different ligands, we prepared a series of ER-LBDs bound to estradiol, DES, tamoxifen and nonylphenol and collected circular dichroism spectrum. This study allowed us to optimize the conditions for incorporation of weakly bound ligands into the ligand free LBD, as well look for changes in secondary structure in the protein as a function of bound ligand. Ligands were introduced into a sample of the ligand free ER-LBD by dialysis. In all cases, the CD spectrum was indicative of highly helical states. This leads us to two conclusions (1) dialysis is a good strategy for preparation of complexes and (2) the structure differences between ligand bound states is subtle and not detectable by circular dichroism, which reports primarily on secondary structure state. This conclusion is consistent with the available crystallographic data, and highlights the need for a high-resolution method (such as NMR) for understanding the structural response of the ER to different small molecules.

Ligand-binding Assay

In order to assess the integrity of our estrogen receptor ligand binding domain (LBD) samples, it is important to have an assay to measure the efficacy of ligand binding. In the past, typical binding assays have used whole cell homogenates (*e.g.*, rat uterine cytosol) as a source of unpurified estrogen receptor. In contrast, our goal is to develop a binding assay that can measure the binding efficacy of the purified LBD and any LBD mutants that are used. This assay should be suitable for all ligands being studied, be functional at the conditions we are using for the structural work, and allow for direct comparison of the binding affinity of mutant proteins. Work is presently on-going in the lab in order to develop such an assay. Several published and unpublished protocols are being tried to determine which one will give the most reliable binding assay. Typical binding assays involve the use of a tritiated ligand such as ^{3}H-estradiol and then competing off the tritiated ligand with cold unlabeled ligand. Then, the complex of bound ligand and estrogen receptor needs to be separated from unbound ligand. Typically, the bound complexes are bound to hydroxyapatite and then the bound ligand is eluted with ethanol. The ethanol elution is counted using liquid scintillation counting and the % bound ^{3}H-estradiol is detemined. The amount of ^{3}H-estradiol bound in the absence of any unlabeled ligand (after correction for any nonspecific binding) is taken as the 100% bound quantity and the relative efficacy of ligands at competing off the labeled estradiol is determined. In our case, the ligand binding domain does not appear to be binding to the hydroxyapatite and we are presently

searching for other techniques for distinguishing bound from unbound ligand. It appears that the use of controlled pore glass (CPG) beads, as recommended by our collaborators the Greene lab at the University of Chicago Medical School, is an effective strategy.

ER-LBD Binding to BRCA1

Recently, two exciting advances in the understanding of BRCA1 have been made. First, a region of BRCA1 that interacts with the estrogen receptor has been identified. In fact, this region includes the ligand binding domain, and studies are currently in progress to determine if the ligand binding domain is necessary and sufficient for this potentially very exciting link between BRCA1 and ER. Second, the structure of the complex of BRCA1 and it's biological partner has been determined. Since we have optimized the protocol to produce recombinant ER-LBD suitable for high resolultion structural studies, we are pursuing a collaboration to study the effect of binding the ER-LBD to BRCA1 by NMR. This effort is somewhat preliminary, but may be extremely important if the ER-LBD region is linked to BRCA1 function.

Investigation of Estrogenic Effects of DDT

During the course of this research, we became interested in the estrogenic properties of the pesticide dichlorodiphenyltrichloroethane or DDT, which was in widespread use in the United States from the 1940s until it was banned in the early 1970s. In both cellular and organismal studies, DDT acts as an estrogenic compound. As a result of these findings, many investigators have asked whether there is a possible connection between DDT exposure and breast cancer. However, DDT itself is a very poor estrogen, and only weakly binds the estrogen receptor *in vitro*.

Many studies over the years have examined the possible connection between environmental pollutants and the development of breast cancer. Recently, the Journal of the National Cancer Institute published the combined analysis of five studies evaluating the association of levels of DDE and PCBs in blood plasma or serum with breast cancer risk. We noticed that, in these studies, DDE, a metabolite of DDT, is used as a marker for DDT exposure. When correlated to DDE levels, the majority of studies published to date do not support the hypothesis that elevated exposure to DDT increases the risk of breast cancer. We wondered, however, whether residual DDE levels are an appropriate marker for determining the estrogenic component of DDT exposures.

A substantial body of research indicates that it is **not** chemicals like DDT or DDE (which contain a chlorinated phenyl ring)(Figure 12) that are estrogenic compounds and therefore potentially carcinogenic. Rather, compounds capable of triggering an estrogenic response typically contain a hydroxylated phenyl ring, a configuration that allows the chemical to bind and activate the estrogen receptor. It should be considered, therefore, that it is not DDT or DDE themselves, but hydroxylated metabolites of these compounds, that are acting as estrogenic compounds and that are then being cleared from the system.

Studies spanning over six decades have definitively established the importance of a hydroxylated phenyl ring for estrogen receptor binding and estrogenic activity. For example, an extensive analysis of structure-activity relationships (SAR) for estrogen receptor ligands published in 1999 concluded that, "A phenolic hydroxy group, which mimics the 3-OH on the A-

ring of estradiol (and at the corresponding position of nonsteroidal estrogen ligands), appears repeatedly as the most important factor in receptor-ligand interactions.".

The importance of a hydroxylated phenyl ring for estrogenic activity is reinforced by the crystal structures of the ligand binding domains of both estrogen receptor α (ERα) and estrogen receptor β (ERβ) published in recent years. All of the structures indicate that the key phenolic hydroxyl of the bound ligands is hydrogen bonded to a highly conserved arginine and a glutamic acid in the ligand-binding domain (*e.g.* arginine 394 and glutamic acid 353 in ERα). This hydrogen bonding interaction acts as a clamp on the phenolic hydroxyl and it is unlikely that the chlorinated phenyl ring of DDT or DDE is able to fulfill this interaction.

We also note that, for a given exposure, an individual with a more active hydroxylation response (*e.g.* through the cytochrome P450 system) would have a **greater** exposure to hydroxylated (and presumably, therefore, estrogenic) metabolic products, but a **lower** residual level of DDE. For all of these reasons, we have questioned whether using residual levels of DDE is an accurate marker to use when assessing the estrogenic consequences of DDT exposure.

We are quite surprised that this point has not been brought out in the literature to date, and have initially pointed this out in a letter to the editor, which has been accepted at the Journal of the National Cancer Institute. We have prepared a more complete description of these concerns as a review article, which is currently under review.

Pressure Refolding Studies

We are using high-throughput biophysical strategies to probe the conformational response of the ER-LBD to a variety of estrogens, antiestrogens and environmental estrogens. A critical problem in the preparation protocol is the isolation and refolding of the protein. When recombinant proteins are produced in prokaryotes such as *E. coli*, improper folding and secretion of the overexpressed protein frequently results in misfolded, nonnative protein aggregates.

To address this challenge, we have developed a new strategy to refold active protein at high yield using a novel technique that applies high hydrostatic pressures to disaggregate and refold proteins. This revolutionary new strategy, pioneered by Prof. Randolph's laboratory at CU, is superior to current techniques because it yields high concentrations of biologically active proteins, without requiring the addition of exogeneous chemicals that may adversely affect subsequent experiments or necessitate additional purification. This methodology is being developed in Prof. Randolph's laboratory in collaboration with the pharmaceutical and biotechnology industries to increase the yield of a number of pharmaceutically important proteins, and dramatic enhancements of yield have been attained in several systems. The ER-LBD is an ideal candidate for pressure refolding given that much of the protein expresses as aggregates that can be disrupted with mild concentrations of urea and detergent. Protein that has been refolded using the pressure strategy will be rigorously tested for activity using a binding assay to various ligands whose binding properties are well known. We have begun pilot refolding studies, and have obtained future funding for this project from the Butcher Foundation.

KEY RESEARCH ACCOMPLISHMENTS

- Highly purified, stable LBD-ER has been prepared and conditions for expression with a variety of NMR-active isotope labels have been optimized.
- Analysis of the differences between ^{15}N-^{1}H HSQC NMR data on estradiol and tamoxifen complexes has been initiated.
- The feasibility of obtaining resonance assignments at our highest available field strength has been assessed.
- We have obtained funding for 800 and 900 MHz spectrometers to greatly enhance our ability to continue to use NMR to understand the structural response of the ER-LBD.
- A new protocol for the preparation of ligand-free ER has been developed and evaluated.
- Thermodynamic analysis of protein/ligand complex stability has been conducted.
- A binding assay to determine activity at our experimental conditions in a systematic fashion is currently being developed.
- CD characterization has been performed with different ligand-bound states of the protein
- A collaboration to investigate the BRCA1 interaction with the ligand free and ligand bound ER-LBD has been initiated
- Observations regarding the possibility of hydroxylated metabolites of DDT as hormone mimics and relationship to of breast cancer have been made and published.
- A novel protein refolding strategy using pressure refolded has been initiated. These studies will be funded in the future by the Butcher Foundation.

REPORTABLE OUTCOMES

Papers published:

L. W. Glustrom, R. M. Mitton-Fry and D. S. Wuttke, "Environmental Estrogens and Breast Cancer: The Importance of Hydroxylated Intermediates," reviewed letter to editor, *J. Natl. Cancer Institute*, **2002**, *94*, 68-69.

L. W. Glustrom and D. S. Wuttke, "DDT as an Environmental Estrogen: The Need to Consider Hydroxylated Metabolites," submitted.

Posters presented: The work described in this progress report has been presented at several meetings as either a talk or poster. These include (1) The University of Colorado Annual Retreat (Winter Park, Colorado) (2) The University of Colorado Biophysics Supergroup Meeting (3) The

EPA-STAR Award Annual Meeting (Washington, DC) and (4) a full report of this work was presented at the Era of Hope 2002 Department of Defense Breast Cancer Research Meeting in Orlando, Florida, September of 2002.

Graduate Student Training: Two graduate students, Dana Warn and Aaron Miller, have been trained on this research project. Dana Warn has been primarily responsible for protein expression and purification protocols, and NMR spectroscopy. Her stipend and tuition were provided through an EPA-STAR fellowship. During the last year of funding, **Dana Warn** obtained a Master's Thesis in Chemistry and Biochemistry from the University of Colorado entitled "Environmental Estrogens and Breast Cancer Therapeutics: Development of a Technique to Characterize the Diverse Ligand Binding Characteristics of the Estrogen Receptor," awarded December, 2000. **Aaron Miller** was a first year graduate student who worked on the development of a preparation protocol for the ligand-free estrogen receptor ligand binding domain.

Undergraduate Training: **Andrea Wismann,** an undergraduate at the University of Colorado, Boulder, conducted independent research on this project during the summer of 2000. Through this research experience, she learned protein expression and purification strategies, as well as circular dichroism spectropolarimetry. **Bryn Weaver**, an undergraduate Biochemistry and MCDB major, has joined the project. She has been investigating the properties of the ligand-free ER-LBD. In particular, she has expressed and purified the ligand-free ER-LBD and determined the circular dichroic properties of this state of the protein. Her work in the Summer of 2002 was supported by a UROP grant from the University of Colorado for undergraduate research education.

Postdoctoral Training: Dr. **Aimee Eldridge**, a postdoctoral fellow trained in NMR spectroscopy, optimized and collected data on several ER-LBD complexes, as well as the free state of the protein, for this project.

Professional Research Associate Training: The re-optimization of our protein expression protocol and the development of a ligand binding assay has been performed by **Leslie Glustrom**, a professional research associate in the laboratory who is currently being trained on this project.

CONCLUSIONS

In this project, we set out to use high resolution NMR spectroscopy to understand the conformational changes that occur in the ER-LBD in response to ligand binding. The first part of this effort was successful, and we were able to develop optimized protocols for expression and purification of highly (^{15}N, ^{15}N and ^{13}C, ^{15}N, ^{13}C, ^{2}H) isotopically labeled protein. Preliminary NMR data were collected on all of these samples at our highest available magnetic field. We were able to capitalize on the recent developments in pulse sequence methodology (*i.e.*, TROSY sequences) and determine the optimum liganded state for further characterization. However, the large size of the protein and its unfavorable solution behavior prevented us from making the resonance assignments needed to perform our initially proposed chemical shift mapping experiments.

In response to these challenges, we pursued several strategies. First, we obtained $8 million in funded to establish the NMR resources needed to complete the proposed work. The timescale of funded and building these types of facilities means that the research on ER-LBD is not complete at this time. However, once these resources are in place, we will seek funding to complete these studies. In fact, preliminary data from this research program was a key component in our instrumentation proposals justifying the need for ultra high field instrumentation. In addition, due to the lack of consistent data in the literature, we are developing a ligand-binding assay in our lab to directly compare ligand affinities at the experimental conditions used for structural studies. In parallel, we completed several studies using CD to study ligand binding, as well as developed several new and innovative strategies that allow us to study the ligand free state of the ER-LBD as well as complexes with weakly binding ligands. We also investigated the perplexing issue of the estrogenicity of DDT from a structural perspective, which lead to two publications.

REFERENCES

No references are included in this report.

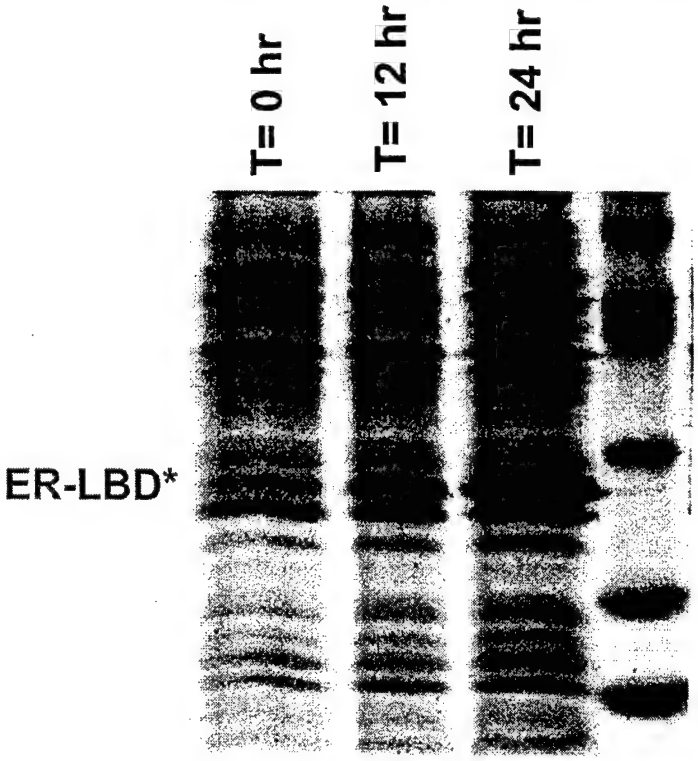
APPENDICES

Figures referred to in the text are appended below.

Figure 1

Production of ER-LBD* for NMR Studies

SDS-PAGE gel of time points after induction



E. coli. (BL21(DE3) pLysS) cells tranformed with plasmid carrying ER-LBD

↓

Grow cells in minimal media in 99% D_2O with $(^{15}NH_4)_2SO_4$ as the sole nitrogen source, ^{13}C glucose as the sole carbon source

↓

Induce with IPTG

↓

Express labeled ER-LBD

Purification using the Estradiol Affinity Column

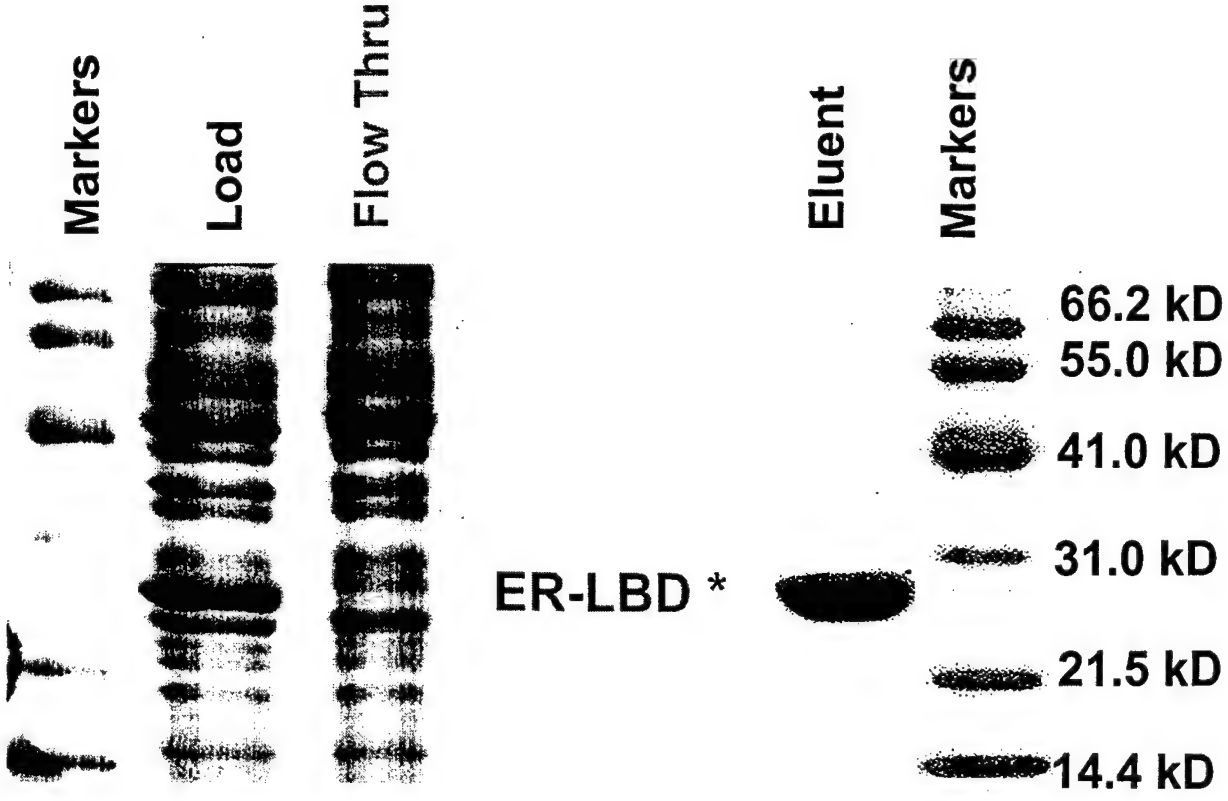


Figure 2

^{1}H - ^{15}N HSQC of ^{15}N ER-LBD Bound to Estradiol
in 20 mM d-Tris, 200 mM NaCl, 1mM d-DTT, 0.02% NaN_3,
Taken at 30° C

The HSQC spectrum of the ^{15}N labeled protein shows the broad linewidths expected for a protein of this size. The HSQC-TROSY technique shown in the following spectrum exhibits reduced linewidths and improved spectral quality.

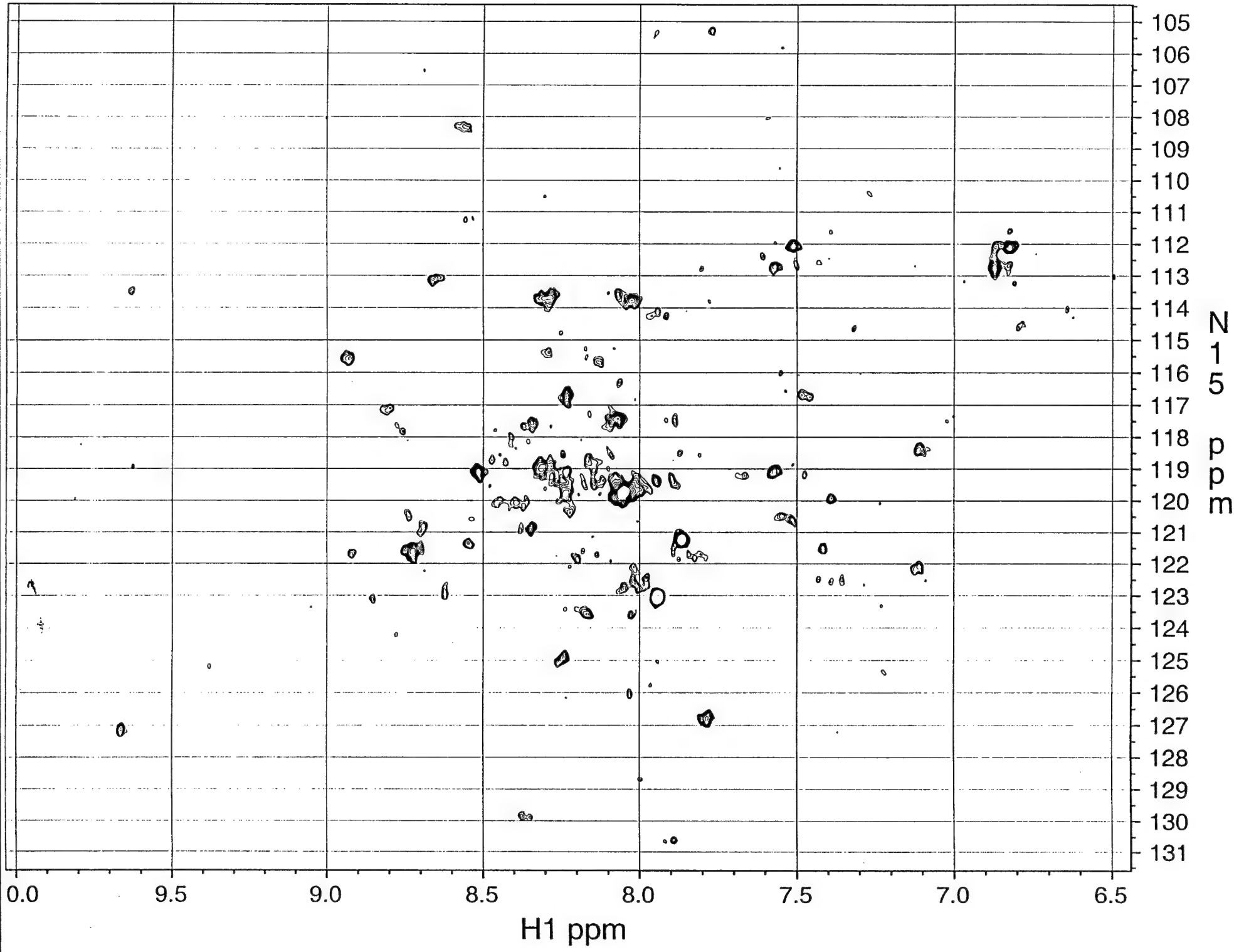
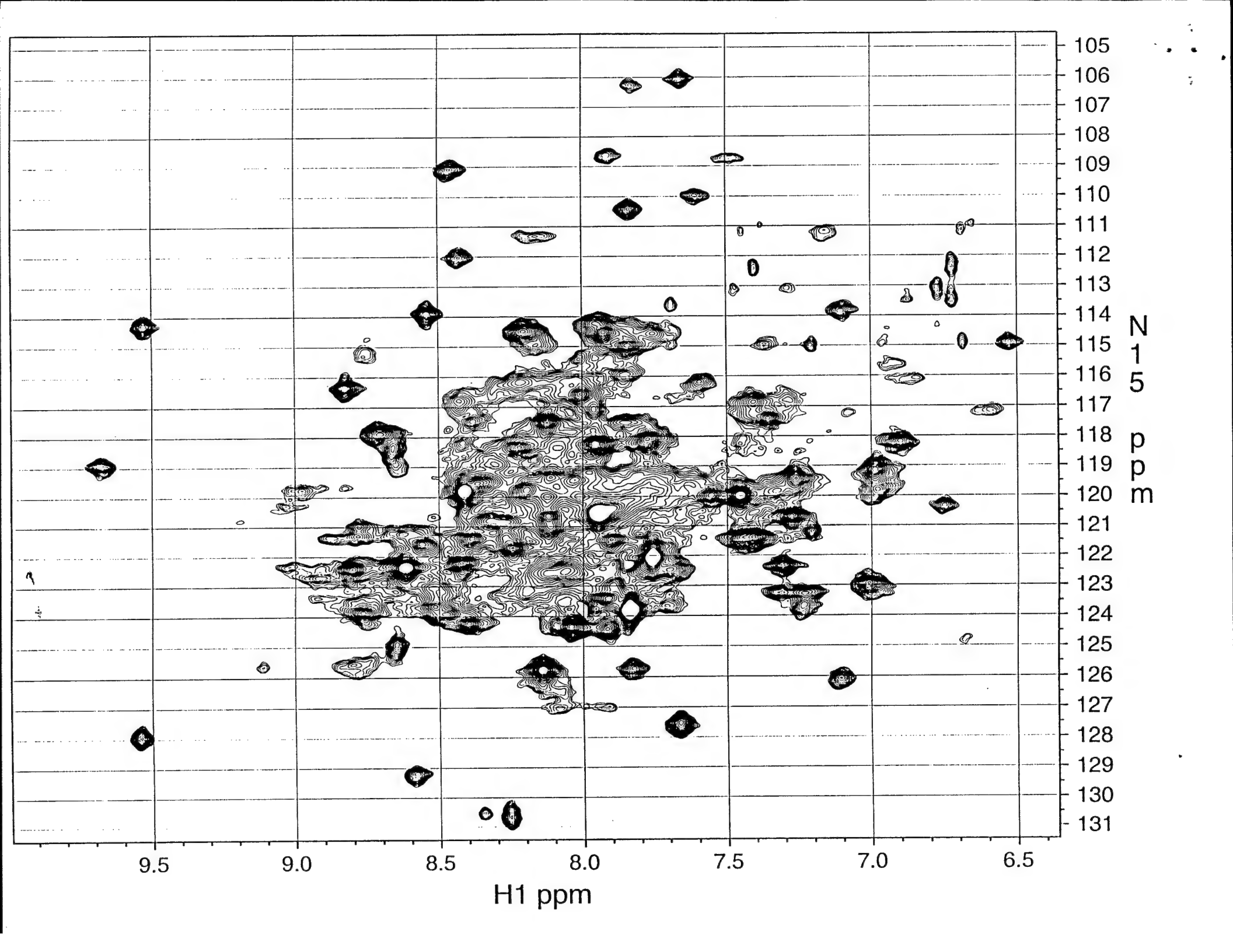

105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
N 15 ppm
0.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
H1 ppm

Figure 3

^{1}H - ^{15}N TROSY-HSQC of ^{15}N ER-LBD Bound to Estradiol
in 20 mM d-Tris, 200 mM NaCl, 1mM d-DTT, 0.02% NaN_3
Taken at 30° C

The HSQC-TROSY spectrum produces a significant improvement in the data acquired, producing narrower linewidths and providing more information than the HSQC experiment. In the HSQC spectrum, the ^{1}H linewidth was 30 Hz; in the HSQC-TROSY spectrum, the ^{1}H linewidth was reduced to 20 Hz. Nonetheless, the TROSY technique alone at 600 MHz does not provide sufficient narrowing of the linewidth for complete resonance assignment.


105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
N 1 5 p p m
9.5
9.0
8.5
8.0
7.5
7.0
6.5
H1 ppm

Comparison of the Estradiol-ER-LBD complex and the 4-OH-Tamoxifen-ER-LBD complex:

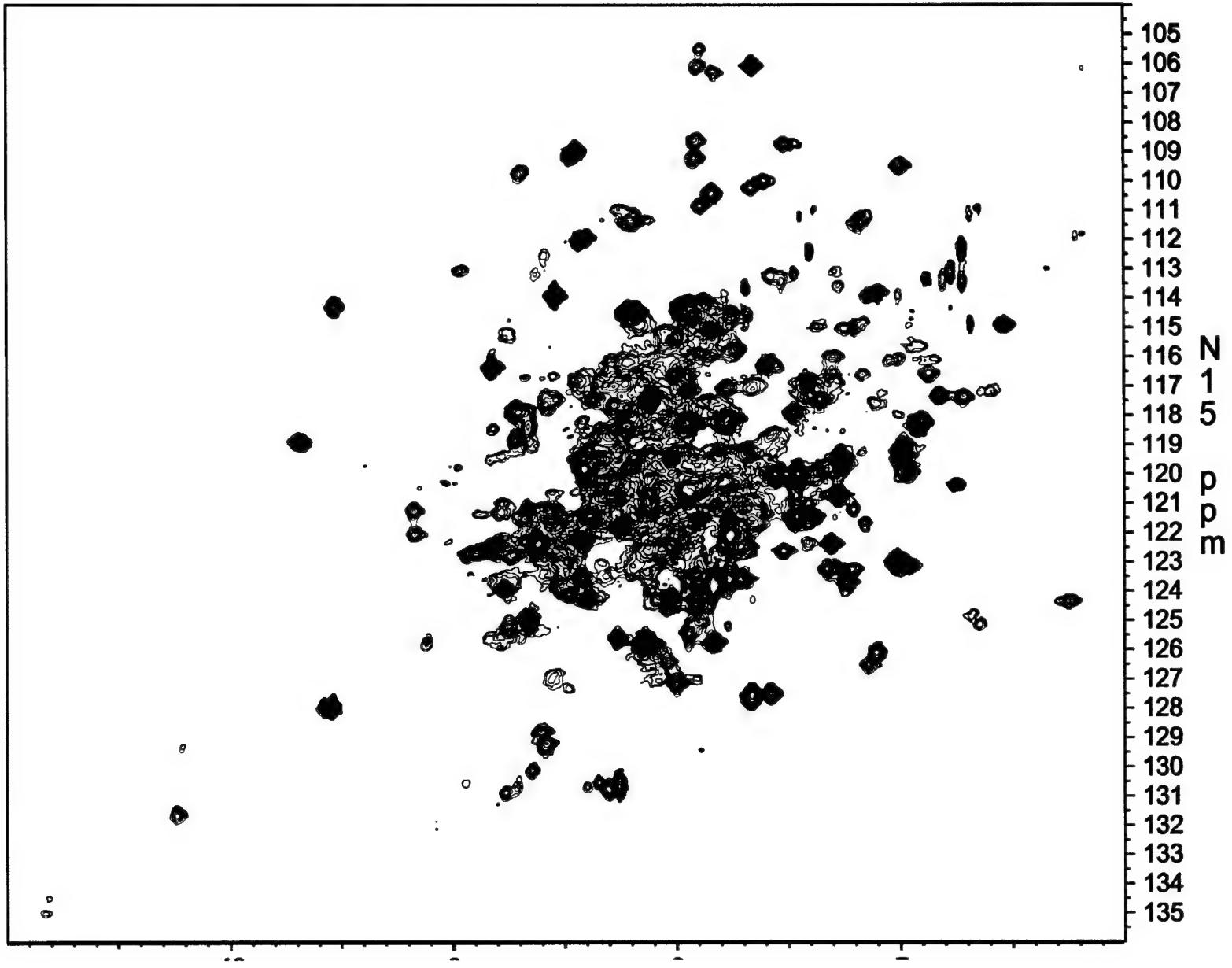


Figure 4

Figure 5

Sample Induction of Partially Deuterated ER-LBD: SDS-PAGE of time points after induction with IPTG

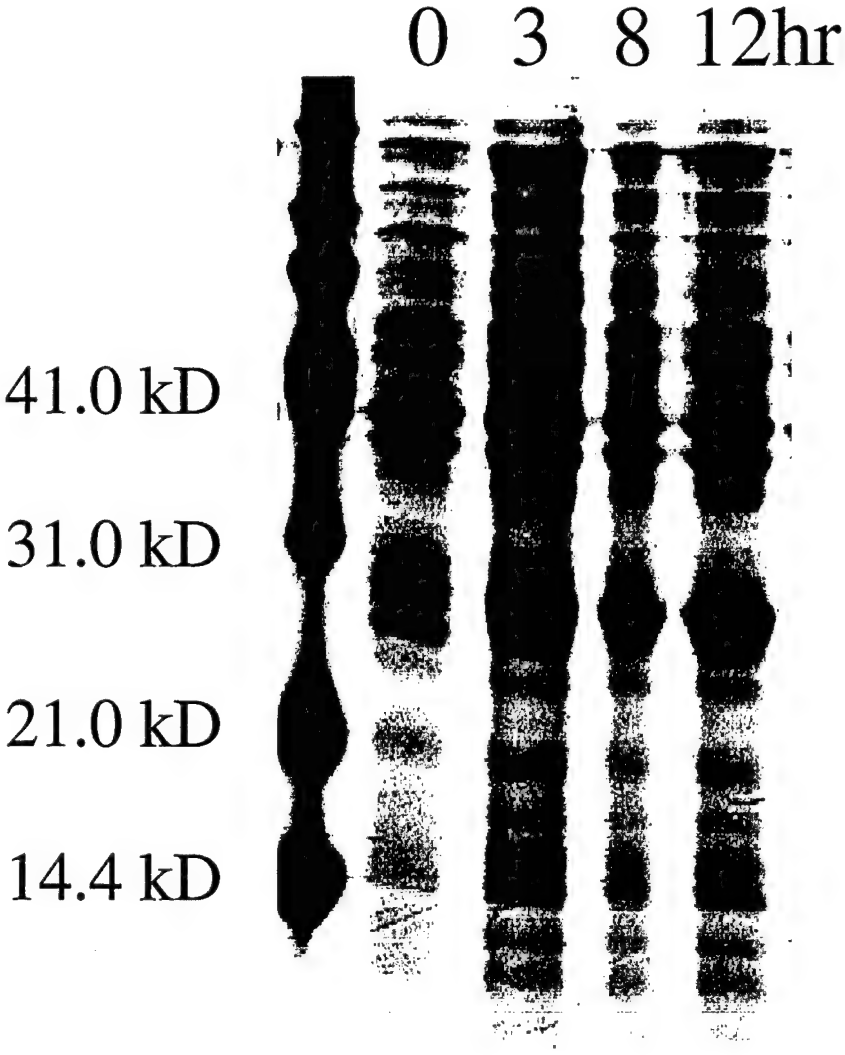
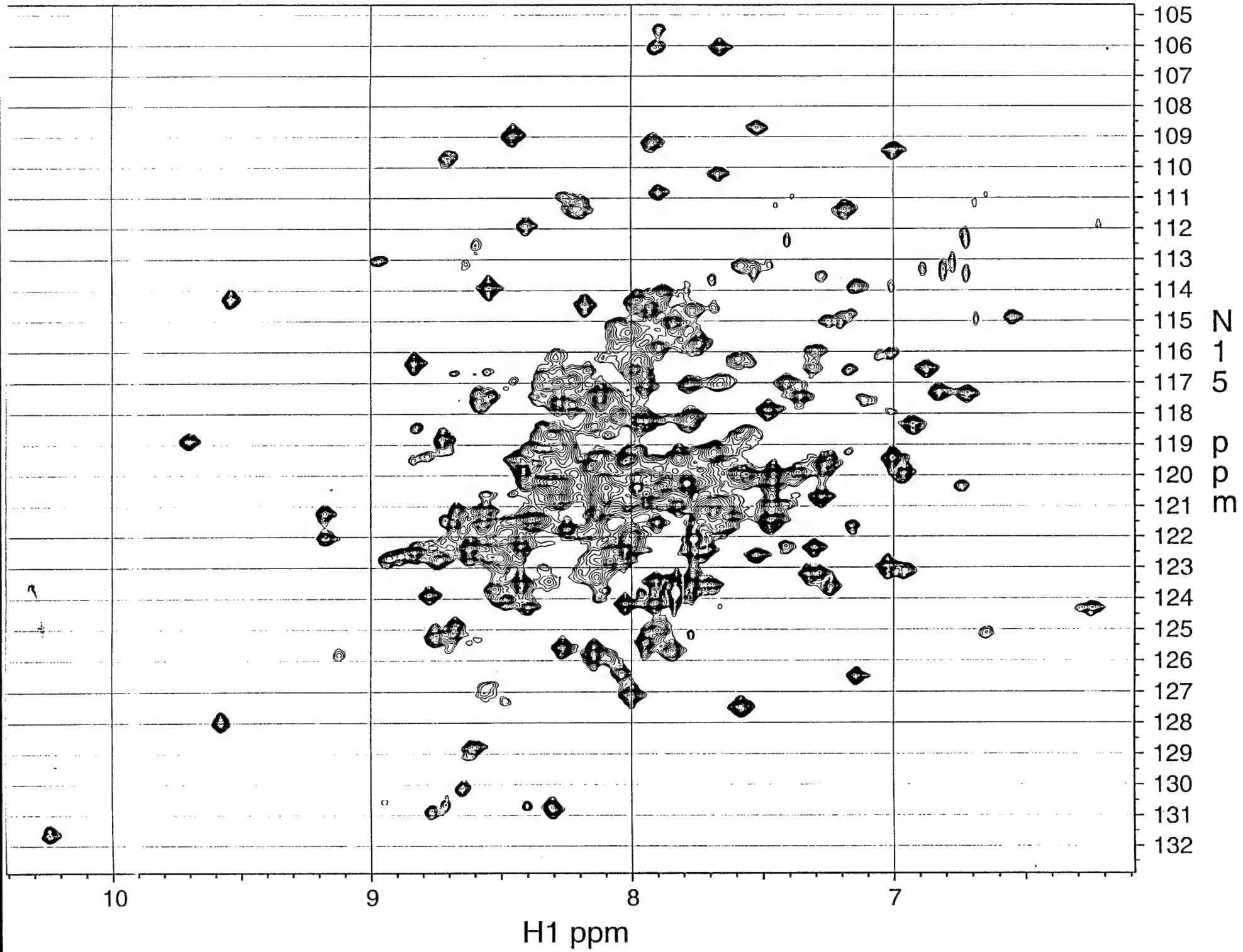


Figure 6

^{1}H - ^{15}N TROSY-HSQC of Partially Deuterated ^{15}N ER-LBD Bound to 4-OH-Tamoxifen in 50mM d-Tris, 100 mM NaCl, 100mM Na_2SO_4, 1mM d-DTT, 0.02% NaN_3, Taken at 30° C

Partial deuteration significantly improves the quality of the data, and will allow for chemical shift mapping. However, it would still not be possible to make full resonance assignments with data taken on a 600 MHz spectrometer. Efforts are now underway to gain access to an 800 MHz spectrometer in order to assess the quality of the data collected at higher field strength.


105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
N
1
5
p
p
m
10
9
8
7
H1 ppm

Test Inductions and Purified His ER-LBD

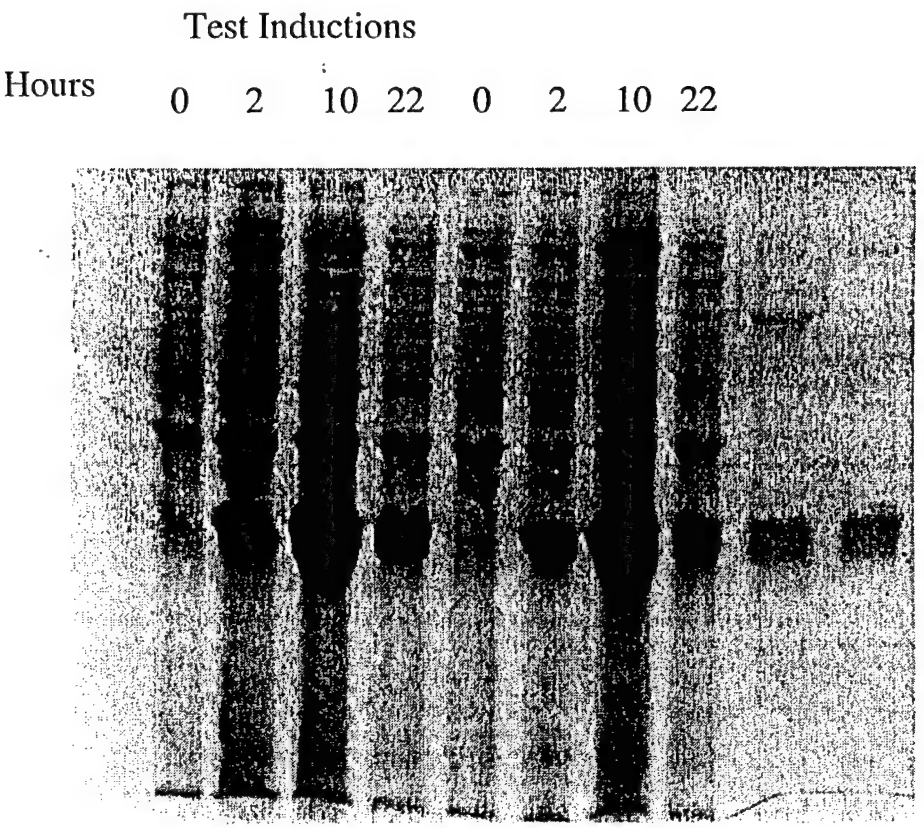


Figure 7

Figure 8

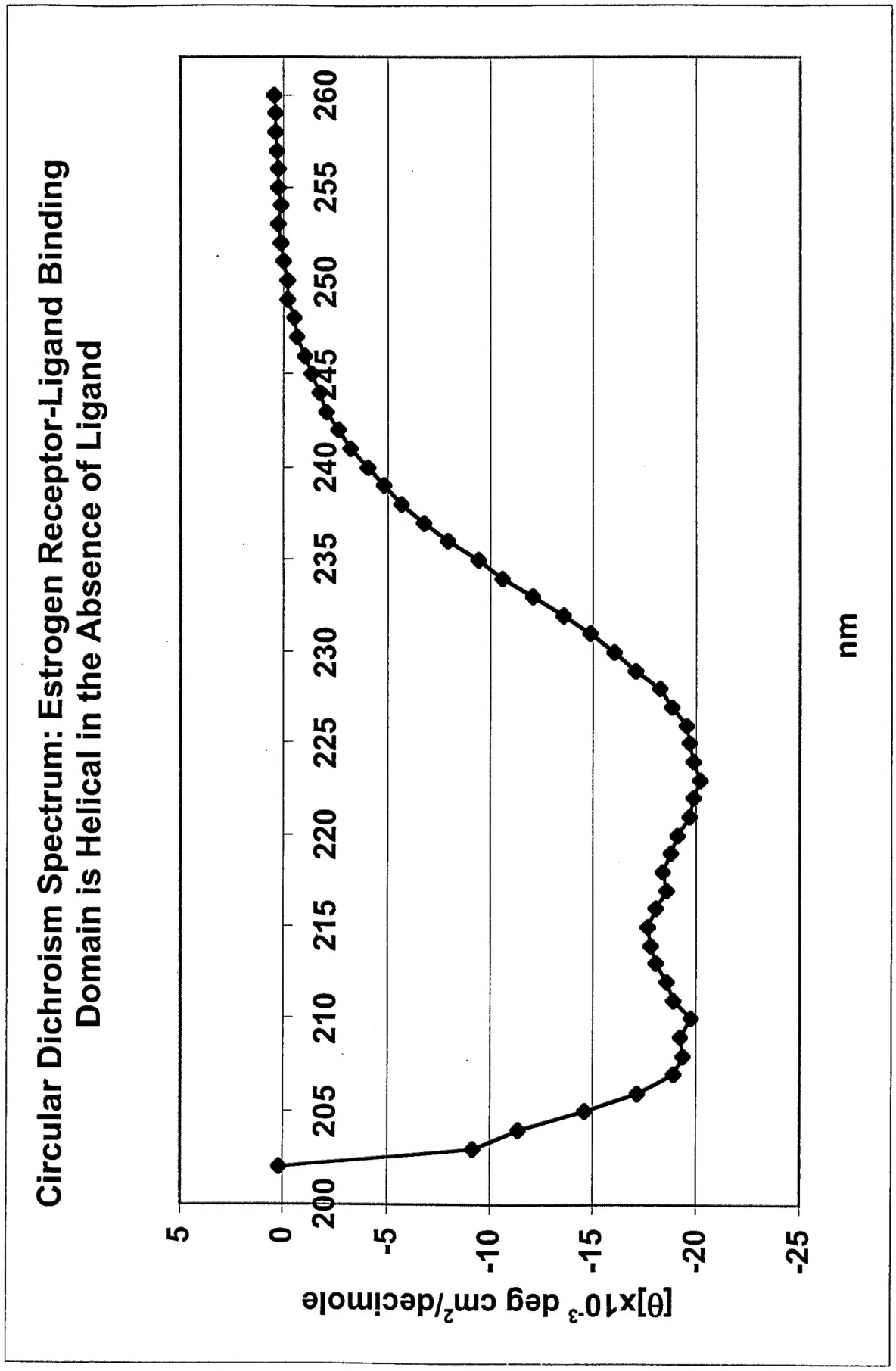
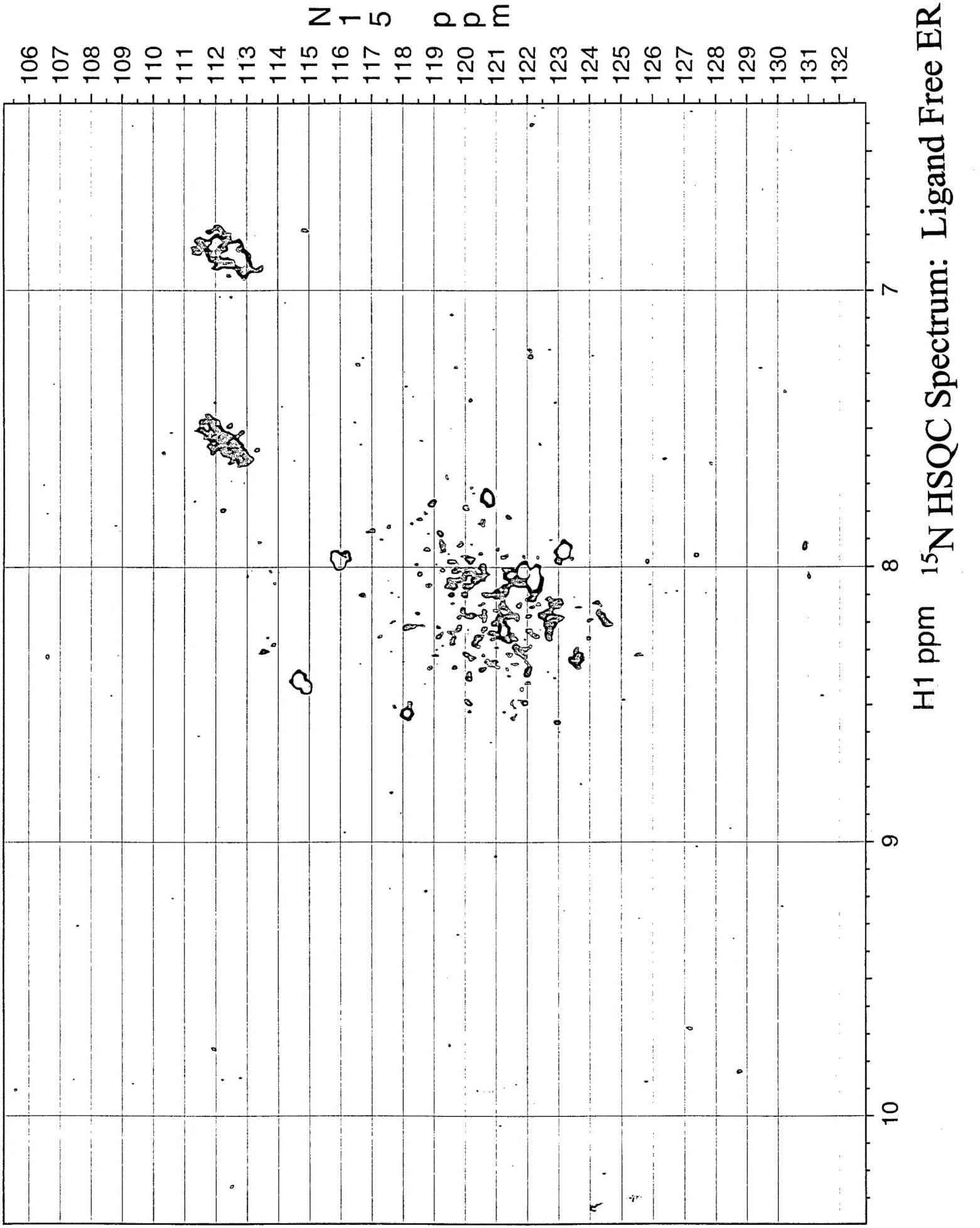

Circular Dichroism Spectrum: Estrogen Receptor-Ligand Binding Domain is Helical in the Absence of Ligand
[θ]x10⁻³ deg cm²/decimole
5
0
-5
-10
-15
-20
-25
200
205
210
215
220
225
230
235
240
245
250
255
260
nm

Figure 9



^{15}N HSQC Spectrum: Ligand Free ER

Figure 10

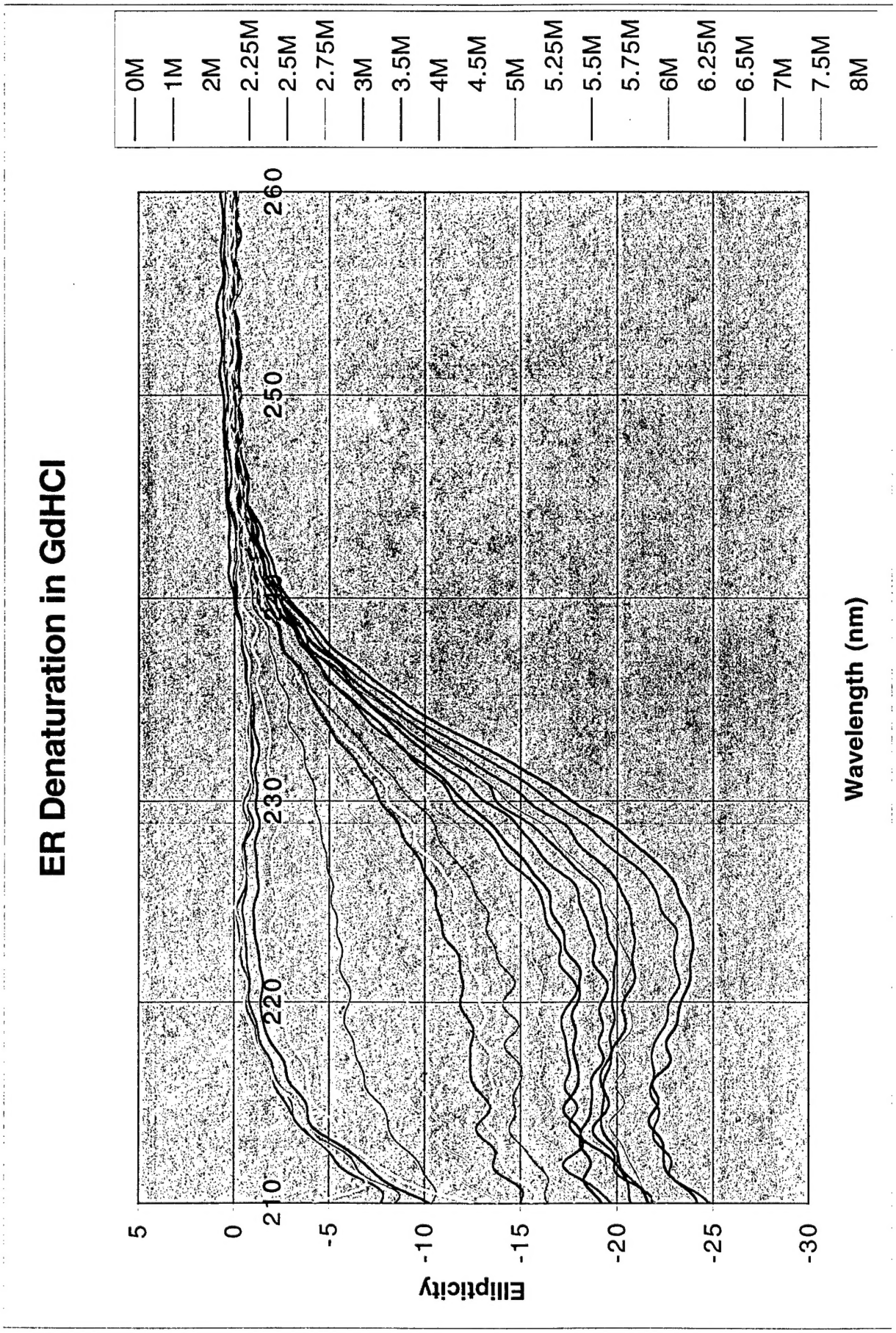
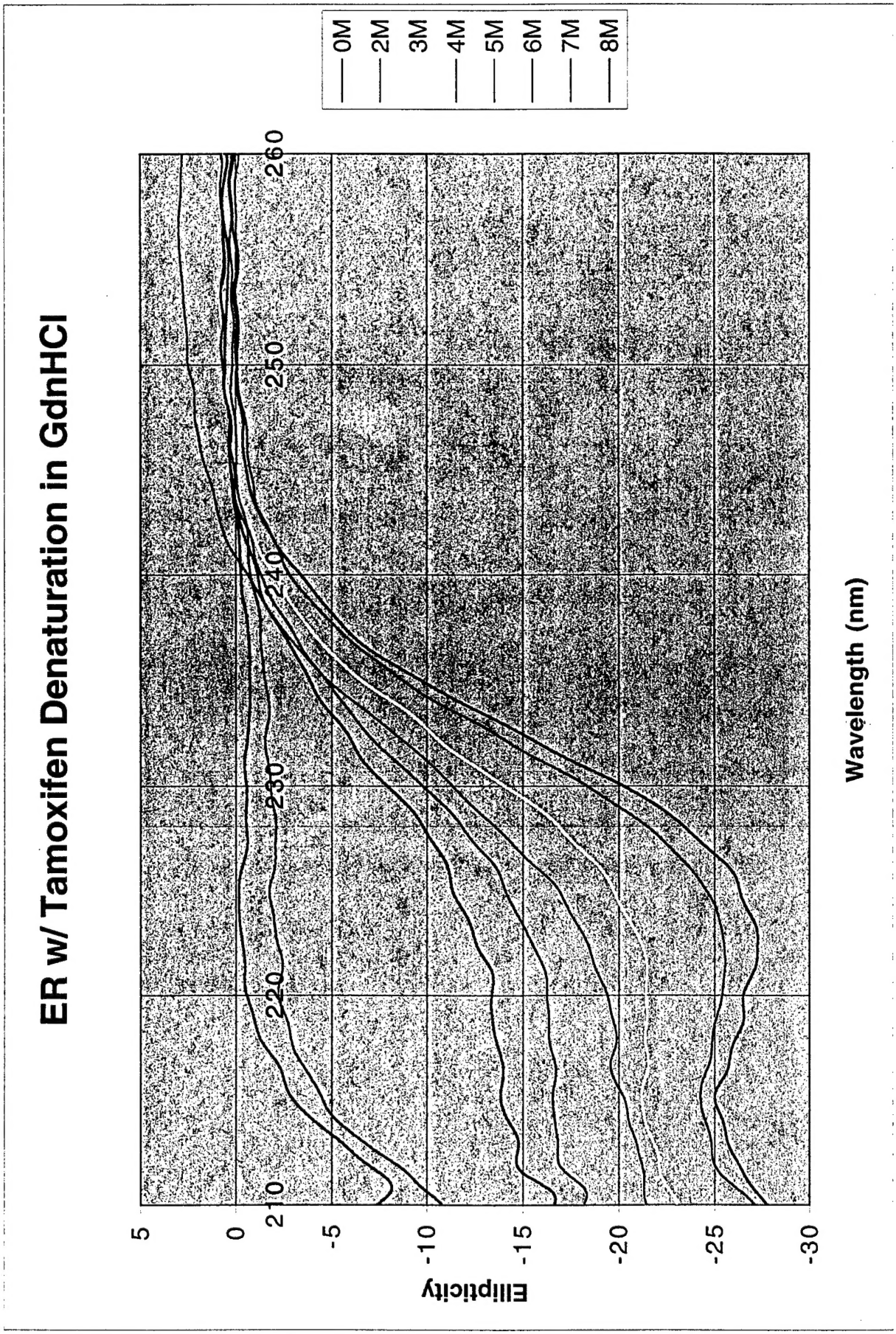

ER Denaturation in GdHCl
0M
1M
2M
2.25M
2.5M
2.75M
3M
3.5M
4M
4.5M
5M
5.25M
5.5M
5.75M
6M
6.25M
6.5M
7M
7.5M
8M
5
0
-5
-10
-15
-20
-25
-30
210
220
230
250
260
Ellipticity
Wavelength (nm)

Figure 11


ER w/ Tamoxifen Denaturation in GdnHCl
0M
2M
3M
4M
5M
6M
7M
8M
Ellipticity
5
0
-5
-10
-15
-20
-25
-30
210
220
230
240
250
260
Wavelength (nm)